

The Essentiality of Folate for the Maintenance of Deoxynucleotide Precursor Pools, DNA Synthesis, and Cell Cycle Progression in PHA-Stimulated Lymphocytes

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The fidelity and progression of DNA synthesis is critically dependent on the correct balance and availability of the deoxynucleoside triphosphate (dNTP) precursors for the polymerases involved in DNA replication and repair. Because folate-derived one-carbon groups are essential for the *de novo* synthesis of both purines and pyrimidines, the purpose of this study was to determine the effect of folate deprivation on deoxynucleotide pool levels and cell cycle progression. Primary cultures of phytohemagglutinin (PHA)-stimulated splenocytes were used as the cellular model. T-cells and macrophages were purified from spleen cell suspensions obtained from F344 rats and recombined in culture. The cells were harvested after a 66-hr incubation with PHA and analyzed for nucleotide levels by reverse-phase HPLC with diode array detection. The proportion of cells in the different phases of the cell cycle was determined by bivariate flow cytometric measurement of bromodeoxyuridine (BrdU) incorporation and DNA content (propidium iodide staining). PHA-stimulated T-cells cultured in medium lacking folate and methionine manifested significant decreases in the deoxynucleotides dCTP, dTMP, dGTP, and dATP relative to cells cultured in complete medium. The reduction in dNTP pools was associated with a decrease in the corresponding ribonucleotide pools. Flow cytometric analysis revealed a 2-fold increase in S and G₂/mitosis (G₂/M) DNA content in PHA-stimulated cells cultured in the medium lacking folate and methionine, which suggests a delay in cell cycle progression. These alterations in DNA content were accompanied by a 5-fold decrease in BrdU incorporation relative to PHA-stimulated cells cultured in complete medium. Supplementation of the deficient medium with folate resulted in normalization of dNTP content and BrdU incorporation. The data are consistent with the interpretation that folate deprivation results in reduced dNTP levels that may retard DNA synthesis and cell cycle progression.

Introduction

The synthesis and turnover of deoxynucleoside triphosphate (dNTP) pools are tightly coupled to DNA synthesis (1). Because folate-derived one-carbon groups are essential for the *de novo* synthesis of purines and pyrimidines, it was of interest to determine the effect of folate deprivation on nucleotide pools, DNA synthesis, and cell cycle progression. In addition to folate,

methionine was omitted from the medium to increase the intracellular folate requirement and further stress folate availability for dNTP biosynthesis (2,3). The irreversible diversion of 5,10-methylene tetrahydrofolate to 5-methyl-tetrahydrofolate for the regeneration of methionine further compromises folate availability for *de novo* nucleotide synthesis (Fig. 1). Since dNTPs are the immediate precursors for the polymerases involved in DNA replication and repair, the fidelity of DNA synthesis is critically dependent on the correct balance and availability of deoxynucleotides (4,5). Several studies have shown that dNTP imbalance *in vitro* induced by antifolate drugs will promote certain genetic (and cancer-associated) lesions including folate-sensitive fragile site expression (6,7), DNA strand breakage (8), error-prone DNA repair (9), and mutagenesis (10). In the present study we attempted to simulate *in vivo* condi-

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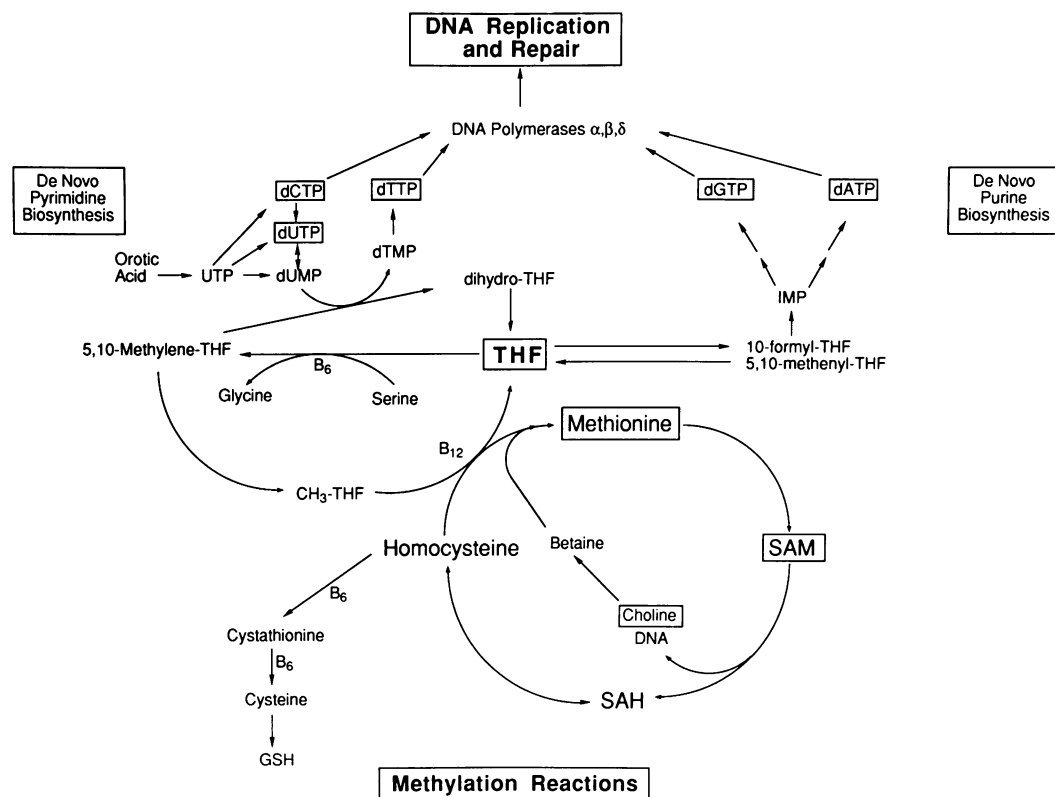


FIGURE 1. Graphic representation of one-carbon metabolism demonstrating the interaction between tetrahydrofolate (THF) and methionine. Methionine deprivation increases the requirement for folate by stimulating the folate-dependent methylation of homocysteine to regenerate methionine. The diversion of folate toward methionine resynthesis reduces folate availability for nucleotide biosynthesis.

tions of intracellular folate and methionine deficiency by omitting these essential nutrients in the medium of cultured splenocytes. Lymphocytes cultured *in vitro* can be stimulated to proliferate via mitogen exposure, and they provide a convenient model for the study of aberrant DNA metabolism and cell cycle progression under conditions of folate deprivation. The results of this study indicated that folate/methionine deprivation in phytohemagglutinin (PHA)-stimulated T-cells is associated with a decrease in intracellular nucleotide pools, an arrest in DNA synthesis, and a delay in cell cycle progression. These alterations did not occur when folate and methionine were added to the deficient medium.

Methods

Spleen Cell Suspensions

Spleens were aseptically removed and single-cell suspensions prepared by gentle teasing with forceps and aspirations through a 21-gauge needle in Medium 199 (Gibco, Grand Island, NY). The suspension was processed for purification of macrophage and T-cell populations. Macrophages were isolated by adherence to petri dishes for 60 min. at 37°C in a humidified CO₂ incubator. The nonadherent cells were decanted and

further enriched for T-cells by elution through nylon-wool columns as described by Julius et al. (11). The plastic adherent macrophages were removed by vigorous washing with Ca²⁺-Mg²⁺-free Hank's balanced salt solution. The viability of both the macrophage and T-cell populations was >95% as determined by Trypan blue exclusion. Purified populations of macrophages and T-cells were recombined in a 1:10 proportion (5 × 10⁶ macrophages: 5 × 10⁷ T-cells) in duplicate 25-mL flasks and cultured in a total volume of 10 mL Medium 199 (M199) plus 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). Aliquots of cells from the same cell suspension were cultured in duplicate in one of three different media: complete M199, deficient M199 lacking in folate and methionine, or deficient M199 supplemented with 1.0 mg/L folic acid and 15 mg/L methionine. Phytohemagglutinin (Sigma Chemicals, St. Louis, MO) was added at 4 µg/mL as a proliferative stimulus. Cells cultured in complete M199 without PHA served as control. After 66 hr, cells were harvested for HPLC and flow cytometric analysis.

Cell Extraction for HPLC Analysis

Cells were transferred to 15-mL tubes and centrifuged at 200 g for 5 min. at 4°C. The supernatant

was removed, and the cell pellet was suspended in 0.5 mL ice-cold 0.6 M trichloroacetic acid (TCA), vortexed vigorously, and kept on ice for 20 min. After centrifugation, the acidic supernatant was transferred to a microcentrifuge tube containing 0.55 mL ice-cold freon-trioctylamine as previously described (12). The mixture was vortexed for 15 sec, and after centrifugation at 4°C, the lower phase was carefully removed by aspiration, leaving the aqueous solution of nucleotides. Samples were lyophilized and subsequently stored at -70°C. Just before analysis, cell extracts were resuspended in 100 μ L of 0.2 M ammonium phosphate, pH 5.35, and filtered through a 0.4- μ m microfuge filter unit. The injection volume was 20 μ L.

HPLC Chromatographic Conditions

HPLC analyses were performed on a Beckman System Gold HPLC system consisting of a programmable solvent module (Model 126) and a diode array detector (Model 168) with an Econosphere C-18 reverse-phase column (5 μ m, 300 \times 4.6 mm, Alltech Associates). Absorbance was monitored at 260 nm using diode array detection. Two buffers were used: buffer A consisted of 0.2 M $(\text{NH}_4)_2\text{HPO}_4$ in 1.0 M KCl, pH 5.35, and buffer B was made of 0.2 M $(\text{NH}_4)_2\text{HPO}_4$ with 1.25 M KCl and 10% methanol, pH 5.0. Buffers were prepared fresh daily in double-distilled water, filtered (0.2- μ m filter, Alltech Associates) and degassed by helium purging before use. Samples were eluted isocratically at a flow rate of 0.8 mL/min with 100% buffer A for 8 min. At $t = 8$ min, a 15-min linear gradient to 80% buffer B was initiated. At $t = 35$ min, the system returned to 100% buffer A (over 0.2 min) to begin equilibrating for the next sample. A 15-min re-equilibration period was required between samples. The identity of dNTP peaks in cell extracts was verified by comparing retention times with dNTP standards and by examining the increase in peak area after quantitative dNTP addition. Peak purity was confirmed by diode array detection. Quantitation of pool sizes was accomplished using individual calibration curves for each nucleotide and Beckman System Gold Software.

Bivariate Flow Cytometric Analysis of BrdU Incorporation and DNA Content

Cultured cells were pulse labeled with 10 μ M bromodeoxyuridine (BrdU) for 60 min at 37°C. Those cells actively synthesizing DNA during this interval will incorporate BrdU into their DNA. After BrdU exposure, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody and propidium iodide exactly as described by the vendor (Becton-Dickinson, San Jose, CA). Control samples were similarly processed but without prior exposure to BrdU. Flow cytometric analysis of individual nuclei (10,000/sample) was conducted using a

FACScan flow cytometer (Becton-Dickinson) equipped with an argon laser tuned to 488 nm. List mode data were converted to a bivariate display of FL1 versus FL2 Fluorescence (BrdU-FITC versus propidium iodide). The percentage of cells in G₁, S, and G₂/M phases of the cell cycle (propidium iodide) and those actively incorporating BrdU were calculated using the Lysys II program from Becton-Dickinson. The macrophage population was excluded from analysis by gating on the DNA histogram.

Results

Effect of Folate Availability on Deoxynucleotide Pools in PHA-Stimulated T-Cells

Figure 2 shows the HPLC profiles of nucleotide pools obtained from cells cultured in *a*) complete M199 without PHA, *b*) complete M199 plus PHA, *c*) deficient M199 (lacking in folate and methionine) plus PHA, and *d*) deficient M199 (supplemented with folate and methionine) plus PHA. Quantitative analysis of pool sizes expressed as pmole/10⁶ cells is presented in Table 1. Relative to PHA-stimulated cells cultured in complete M199, the ribonucleotides CTP, UTP, GTP, and ATP and the pteridine ribonucleotide NAD were consistently reduced in the cells cultured in deficient M199 lacking in folate/methionine. Similarly, the deoxyribonucleotides dCTP, dUTP, dGTP, dTMP, and dATP were found to be reduced in cells cultured in the deficient medium. In contrast, nucleotide levels of cells cultured in the deficient M199 supplemented with folate and methionine were found to exceed those observed in cells cultured in the complete M199.

Effect of Folate Availability on Cell-Cycle Progression in PHA-Stimulated T-Cells

Dual parameter flow cytometric analysis of DNA synthesis (BrdU; FL1) and DNA content (PI; FL2) are presented in Figure 3. The proportion of cells in the various phases of the cell cycle are presented in Table 2. In the cells cultured in complete M199, PHA stimulation resulted in a marked increase in BrdU incorporation as expected (Fig. 3 A,B). However, BrdU incorporation in PHA-stimulated cells cultured in the deficient medium was reduced 5-fold to that equivalent to cells cultured without PHA in the complete medium (Fig. 3A,C). The proportion of PHA-stimulated cells in S and G₂/M in the deficient medium was increased 2-fold compared to PHA-stimulated cells cultured in the complete medium and may reflect a delay in cell cycle progression (Fig. 3B,C). Supplementation of the deficient medium to control levels of folate and methionine allowed the cells to progress normally through the cell cycle with kinetics comparable to cells cultured in complete M199 (Fig. 3B,D).

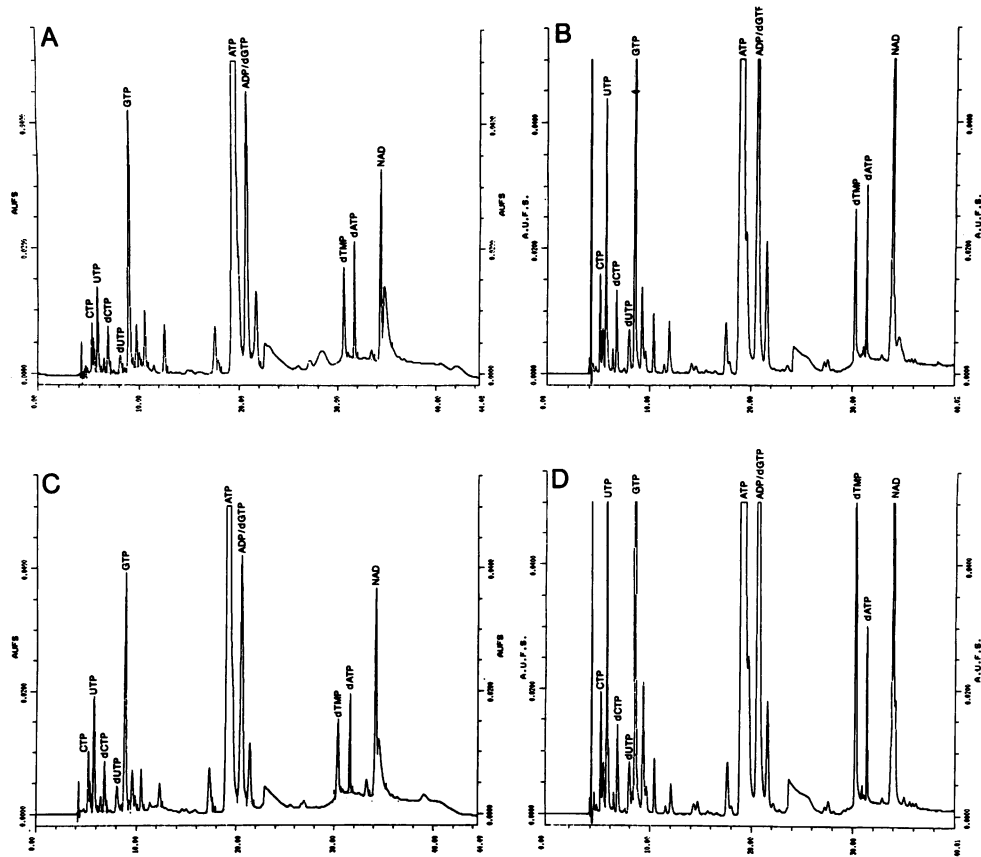


FIGURE 2. Reverse-phase HPLC profile of nucleotide pools in spleen cells cultured (A) without phytohemagglutinin (PHA) in complete Medium 199 (M199), (B) with PHA in complete M199, (C) with PHA in deficient M199 lacking folate and methionine, and (D) with PHA in deficient M199 supplemented with folate and methionine.

Discussion

Agents or conditions that alter the balance of intracellular deoxynucleotides have been previously shown to alter the rate of DNA synthesis and cell-cycle progression. For example, lymphocytes exposed to antifo-

late drugs such as 5-fluorouracil or methotrexate exhibit deoxynucleotide pool imbalance and arrest of DNA synthesis (8,13). Hydroxyurea suppresses replicative DNA synthesis by inhibiting ribonucleotide reductase and depleting dNTP pools (14,15). DNA replication fork movement has been shown to be

Table 1. Nucleotide pools (picomole/10⁶ cells).

	Complete M199 without PHA	Complete M199 plus PHA	Deficient M199 plus PHA ^a	Supplemented M199 plus PHA ^b
CTP	5.8	10.7	8.5	13.0
UTP	13.5	30.8	18.1	42.8
dCTP	11.8	16.0	12.2	20.0
dUTP	5.2	8.6	7.5	11.1
GTP	28.1	36.4	28.8	52.1
ATP	370.2	398.2	330.3	514.0
dGTP	16.2	17.0	11.7	18.0
dTMP	15.8	19.4	13.7	41.8
dATP	10.6	11.6	9.1	13.4
NAD	24.1	40.3	27.3	55.2

Abbreviations: M199, Medium 199; PHA, phytohemagglutinin; CTP, cytidine triphosphate; UTP, uridine triphosphate; d, deoxy; GTP, Guanosine triphosphate; ATP, adenosine triphosphate; TMP, thymidine monophosphate; NAD, nicotinamide adenine dinucleotide.
^aMedium 199 minus folate and methionine.
^bMedium 199 plus folate and methionine.

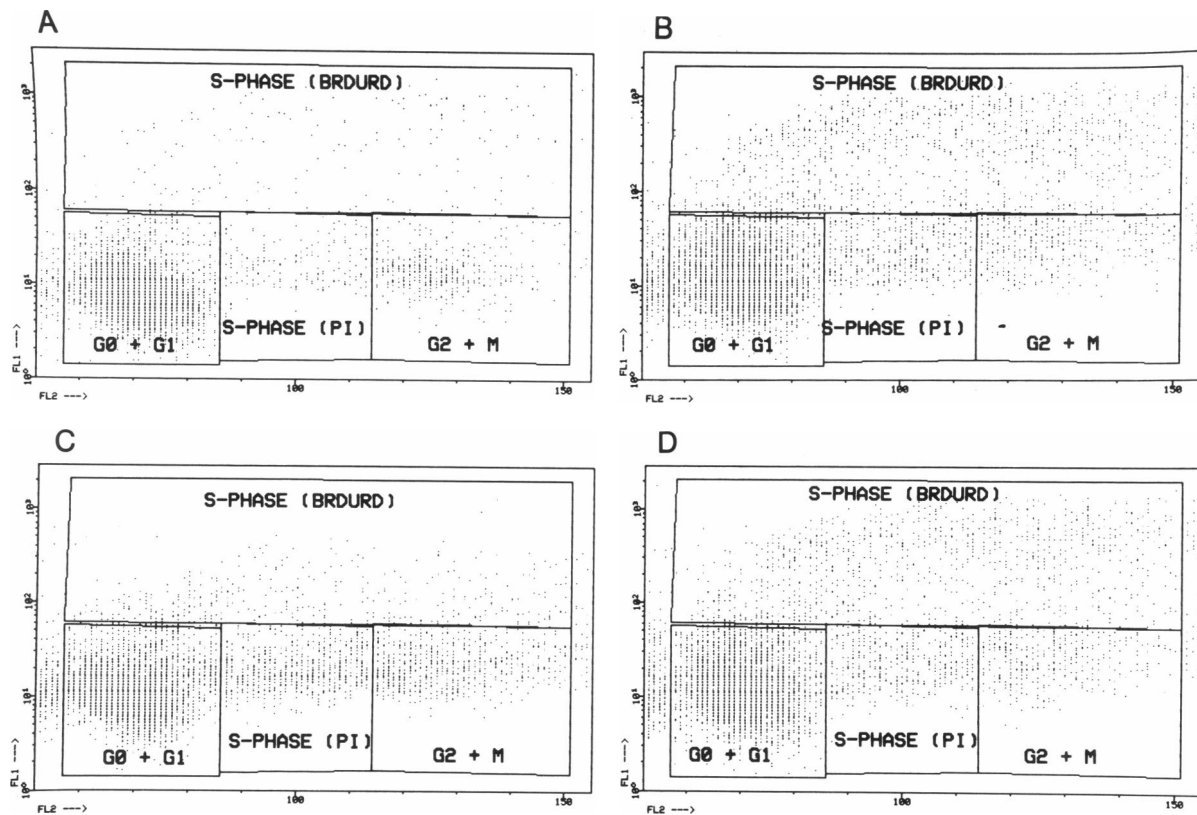


FIGURE 3. Cell cycle analysis of (A) splenic T-cells cultured in complete Medium 199 (M199) without phytohemagglutinin (PHA), (B) splenic T-cells cultured in complete M199 with PHA, (C) spleen cells cultured with PHA in deficient M199 lacking folate and methionine, and (D) spleen cells cultured with PHA in deficient M199 supplemented with folate and methionine.

Table 2. Percentage of cells in G₀/G₁, S, and G₂/M under various culture conditions.

Phase	Complete M199 without PHA	Complete M199 plus PHA	Deficient M199 plus PHA ^a	Supplemented M199 plus PHA ^b
G ₀ /G ₁	64.7	42.1	43.7	48.7
S	2.3	5.7	9.0	4.7
S (+BrdU)	1.2	8.0	1.5	9.2
G ₂ /M	4.9	4.9	8.2	4.8

Abbreviations: M199, Medium 199; PHA, phytohemagglutinin; BrdU, bromodeoxyuridine; M, mitosis.

^aMedium 199 minus folate and methionine.

^bMedium 199 plus folate and methionine.

reduced in human megaloblastic lymphocytes (16) that also exhibit dNTP pool imbalance. In other studies, alterations in dNTP pools have been associated with DNA strand breakage (8), mutagenesis (10,17), and error-prone DNA repair (18,19). In the present study, the effect of folate and methionine deprivation on nucleotide and deoxynucleotide pools and cell-cycle kinetics was evaluated in primary cultures of PHA-stimulated T-cells. The observed decrease in T-cell nucleotide and deoxynucleotide derivatives of adenine, guanine, uridine, and cytidine under conditions of

folate deficiency would suggest a decrease in folate-dependent *de novo* nucleotide synthesis and/or an increase in nucleotide degradation.

The decrease in nucleotide pool levels in T-cells cultured under folate-deficient conditions was associated with a delay in cell-cycle progression and an apparent arrest in DNA synthesis. Taken together, the data presented support the hypothesis that alterations in dNTP pool size may directly or indirectly affect the rate of DNA synthesis and cell-cycle progression.

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